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Original Article

Serologic evidence of human influenza virus infections in swine populations, Cambodia

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Background This study was conducted from 2006 to 2010 and investigated the seroprevalence of influenza A viruses in Cambodian pigs, including human H1N1, H3N2, 2009 pandemic H1N1 (A(H1N1)pdm09), and highly pathogenic avian H5N1 influenza A viruses.

Methods A total of 1147 sera obtained from pigs in Cambodia were tested by haemagglutination inhibition (HI) assays for antibody to human influenza A viruses along with both HI and microneutralization (MN) tests to assess immunological responses to H5N1 virus. The results were compared by year, age, and province.

Results Antibodies against a human influenza A virus were detected in 14·9% of samples. A(H1N1)pdm09 virus were dominant over the study period (23·1%), followed by those to human H1N1 (17·3%) and H3N2 subtypes (9·9%). No pigs were serologically positive for avian H5 influenza viruses. The seroprevalence of human H1N1 and H3N2 influenza viruses peaked in 2008, while that of A(H1N1)pdm09 reached a peak in 2010. No significant differences in seroprevalence to human influenza subtypes were observed in different age groups.

Conclusions Cambodian pigs were exposed to human strains of influenza A viruses either prior to or during this study. The implications of these high prevalence rates imply human-to-swine influenza virus transmission in Cambodia. Although pigs are mostly raised in small non-commercial farms, our preliminary results provide evidence of sustained human influenza virus circulation in pig populations in Cambodia.

Keywords Cambodia, influenza A viruses, pig, serology.

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Introduction

Pigs are considered important intermediate hosts and possible 'mixing vessels' for genetic reassortment of influenza viruses owing to dual susceptibility to both human and animal influenza viruses.[1–3] Consequently, pigs have frequently been implicated in the emergence of human virus strains as was seen in the recent influenza pandemic where the 2009 H1N1 pandemic (A(H1N1)pdm09) virus contained a unique genome constellation derived from swine influenza viruses (SIVs), namely the classical swine H1N1 lineage, the North American H3N2 triple-reassortant, and the Eurasian 'avian-like' swine H1N1 virus.[4–6] The molecular characterization of the A(H1N1)pdm09 strain revealed indirect evidence that pigs play a role in the ecology and emergence of influenza viruses.[7] However, there has been no direct evidence that pigs were involved in the epidemiology or spread of pandemic influenza virus in humans.[8]

The first case of A(H1N1)pdm09 virus infection in pigs was detected in a Canadian pig farm soon after the virus emerged in humans in April 2009.[9] Thereafter, over 20 countries from five continents formally reported cases of A(H1N1)pdm09 in pigs to the World Organization for Animal Health (OIE).[10–12] The increase in reported cases of A(H1N1)pdm09 in pigs, together with experimental studies by several teams,[13–15] has confirmed that A(H1N1)pdm09 virus can become established in pig populations. Furthermore, repeated detections of genetic reassortment between A(H1N1)pdm09-like viruses and other swine viruses in the United States, Europe, and Asia suggest that a second generation of reassorted

A(H1N1)pdm09 viruses might have been maintained in pigs for a period of time, and a process of adaptation of the A(H1N1)pdm09 virus to pigs might be occurring.[7,13,16–22] The reassorted H3N2 SIVs with A(H1N1)pdm09 viruses have been detected among humans in the United States.[23] Monitoring both human-to-pig and pig-to-human transmissions of influenza A viruses is, therefore, critical to improve our understanding and minimize the likelihood of these events.

In Cambodia, nearly 70% of all pigs are raised in small-scale farms.[24] Pigs are bred traditionally, cohabit with human under free-range conditions, and are mainly raised to be sold for meat after relatively short periods (10–12 months).[25] Only few commercial piggeries exist, mainly located near Phnom Penh City to supply the high urban demand for pork and other pig products.[26] The domestic pig producers cannot satisfy the demand for pork in the country.[27] It is estimated that approximately 1000 head of pigs or pig carcasses are imported each year from neighboring countries such as Thailand and Vietnam.

Many different influenza subtypes (including H4, H5, H6, H7, H9, H11, and H12) have been isolated from poultry and pigs in Asian countries, where the pig densities are highest worldwide.[28] The scientific communities and the international organizations like WHO, OIE, and FAO agree that influenza surveillance activities around the world are urgently needed, especially in Southeast Asia where only few countries provided data on influenza in swine.[6,29–31] In Cambodia, integrated production systems, consisting of one or more animal species with crops and fish, are predominant,[32] facilitating transmission of influenza viruses from humans-to-swine, swine-to-human, or between pigs and avian species. Influenza viruses are, therefore, suspected to circulate actively, and the generation and dissemination of new variants are a real possibility in Cambodia.

A preliminary study for the detection of influenza A viruses in pigs was carried out by collecting nasal swab samples on a weekly basis from slaughtered pigs in Phnom Penh between 2006 and 2008 (Institut Pasteur in Cambodia, unpublished data). However, among 1000 samples, no influenza viruses were isolated. In addition, no flu-symptom was recorded suggesting that farmers prefer not sending sick animals to abattoirs, probably to avoid investigations from animal health services. Owing to the previous study results as well as concerns about the potential serologic cross-reactivity between A(H1N1)pdm09 and H1 SIVs in pigs,[33] detection of antibodies against SIVs was not performed in this study. Serological surveillance for influenza viruses was, therefore, conducted in Cambodian pigs for the detection of antibodies against human H1N1, human H3N2, human A(H1N1)pdm09, and avian H5N1 viruses.

Methods

Serum samples

A total of 1147 serum samples collected from pigs in Cambodia between 2006 and 2010 were tested for influenza viruses at the Institut Pasteur in Cambodia (IPC) (Figure 1). The sera comprised stored serum specimens from a repository at the National Veterinary Research Institute (NaVRI) of Cambodia and samples collected by the IPC from a slaughterhouse in Phnom Penh. All samples were tested by hemagglutination inhibition (HI) assays for detecting antibodies against seasonal human H1N1 and H3N2 influenza viruses. The 372 serum specimens collected from 2009 to 2010 were additionally tested for A(H1N1)pdm09 virus. Also, 150 samples were selected randomly and tested further for antibodies against avian influenza H5N1 virus. Data on gender, province of origin, and date of sample collection were recorded for all 1147 animals tested. Data on age were recorded except for animals sampled in the slaughterhouse.

Reference viruses

Each sample was tested against the reference strain per subtype from the year of sampling of that specific sample. Human and avian influenza A viruses circulating in Cambodia during the year of sampling were chosen as reference viruses in this study (Table 1). All reference viruses were extracted from the repository of the Virology Unit/National Influenza Centre at the IPC. Assays using H5N1 virus were conducted under biosafety level 3 conditions.

HI assay

A total of 1147 serum samples were tested by HI test. HI assays have been commonly used to detect the presence of antibody to the HA of influenza viruses in animal and human sera. Before testing, the samples were treated with receptor-destroying enzyme (RDE; Denka Seiken Co. Ltd., Tokyo, Japan) to remove non-specific hemagglutination inhibitors, incubated in a water bath at 37°C overnight, and heated in a water bath at 56°C for 30 minutes to inactivate RDE. The RDE-treated sera were then mixed with 1 drop of 2% red blood cells (RBCs) diluted to 1:10 with 0·85% NaCl solution. The RDE-treated sera and RBCs were thoroughly mixed together by hand shaking and kept in a refrigerator for 1 hour.

Haemagglutination inhibition tests were performed using 96-well polystyrene, microtiter plates. In each test, positive and negative serum controls were included. Briefly, 50 μl of phosphate-buffered saline (PBS) was added from rows B to H prior to addition of 50 μl of RDE-treated sera from rows A to H. Serial twofold dilutions were made by transferring 50 μl amounts from the first row to successive rows, and in the final row 50 μl was discarded. Antigen

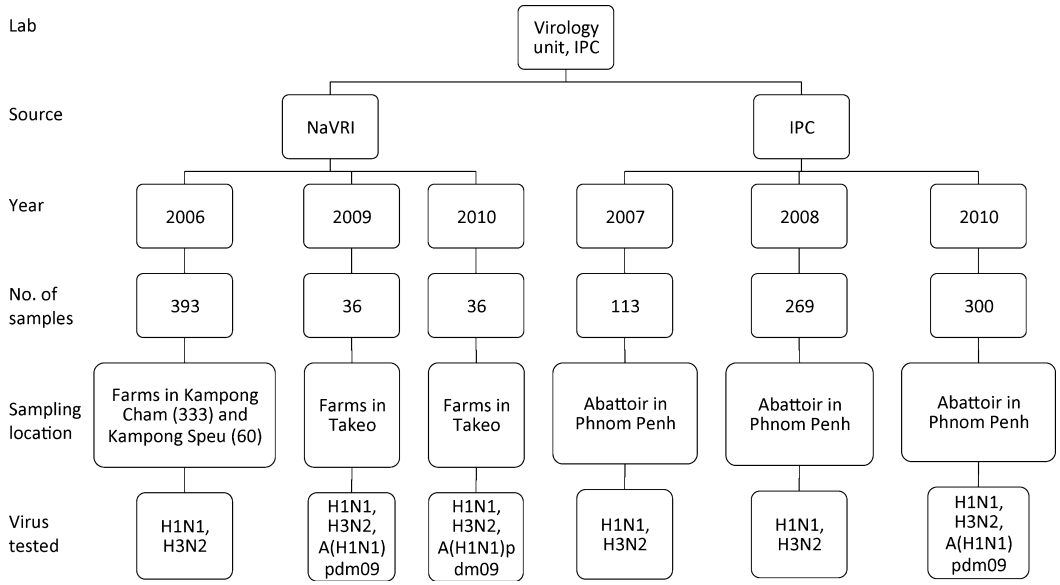

Figure 1. Source of samples and the testing regime.

containing four hemagglutination (HA) units/50 μl of the reference virus was then added to each well and the plates were incubated at room temperature for 15 minutes. Fifty microliters of RBCs were then added to each well. For H1N1 and H3N2 testing, 0·75% guinea pig RBCs was used. For A(H1N1)pdm09 virus, 0·5% Turkey RBCs were used, and for H5N1 virus, 0·5% horse RBCs were utilized (WHO).[34] When using influenza viruses of avian origin, horse blood cells are preferred as they express only receptors to 'avian-type' antigens. For seasonal influenza, we selected the red blood cells type that gave the clearest agglutination with each virus. The plates were incubated at room temperature for 1 hour.

The HI titer was expressed as the highest reciprocal serum dilution that completely inhibited the hemagglutination of 4 HA units of the virus. Considering the previous studies,[35,36] HI titers of 1:40 and higher were regarded to be positive.

Microneutralization assay

The microneutralization test that detects HA subtype-specific antibody is frequently used in parallel with the HI assay for avian influenza virus serology in mammalian specimens.[37] One hundred and fifty serum samples collected from Cambodian pigs and randomly selected were tested for avian influenza antibodies by microneutralization (MN) assay in the BSL3 laboratory of the Virology Unit at the IPC. The MN assay was performed only when the HI titer was ≥20. Briefly, all sera that were already treated with RDE were also heat inactivated at 56°C for 30 minutes. For standard MN assays, 100 tissue culture infectious dose 50 (100 TCID50) of the avian influenza virus, with serial two-fold dilutions of each serum sample (starting from 1:10), were incubated for one hour at room temperature, followed by inoculation of the virus-antibody mixture onto Madin Darby Canine Kidney (MDCK) cells. Cell monolayers were incubated and examined daily for cytopathic effects for 3–4 days. Determining endpoint neutralizing antibody titers was carried out in four wells per dilution. The neutralizing titer was defined as the reciprocal of the highest dilution of serum at which the infectivity of 100 TCID50 of an H5N1 virus for MDCK cells was completely neutralized in 50% of the wells. The titer was calculated by the Reed and Muench method.[38] A seropositive specimen to avian H5 virus was defined by HI and MN titers against H5N1 virus ≥40.[36]

Data analysis

The seroprevalence was calculated along with the 95% confidence intervals using the exact binomial method.[39] The mean ± SD of HI antibody titers was calculated. The seroprevalence rates were compared between years, age, and province. Statistical analyses were performed in spss version 17 (SPSS Inc., Chicago, IL, USA). Seropositivity to human influenza viruses between two age groups was also compared by the two-sided Fisher's exact analysis. Animals for which age was not recorded were excluded from the age analysis.

Results

The seroprevalence rates to each human influenza A serotype and to avian H5N1 virus are displayed in Table 2. The

Table 1. Reference viruses used in the serological tests

Influenza virus	Year of sampling	Assay
A/New Caledonia/20/99 (H1N1)	2006–2007	HI
A/Wisconsin/67/2005 (H3N2)	2006–2007	HI
A/Brisbane/59/2007 (H1N1)	2008	HI
A/Brisbane/10/2007 (H3N2)	2008	HI
A/Brisbane/59/2007 (H1N1)	2009–2010*	HI
A/Perth/16/2009 (H3N2)	2009–2010	HI
A/California/7/2009 (H1N1)	2009–2010	HI
A/Cambodia/Q0321176/2006 (H5N1)	2006	HI, MN
A/Cambodia/S1211394/2008 (H5N1)	2008	HI, MN
A/Cambodia/T1218159/2009 (H5N1)	2009	HI, MN
A/Cambodia/U0417030/2010 (H5N1)	2010	HI, MN

HI, hemagglutination inhibition assay; MN, microneutralization assay.
H3N2 virus circulated in human population during all the duration of the study.
*Seasonal H1N1 viruses circulated in Cambodia every year from 2006 until August 2009 when they were progressively replaced by H1N1pdm 2009 virus. Because the pig sera collected early in 2010 could reflect an exposition that occur in 2009, H1N1 virus was included in the panel also in 2010.

overall seroprevalence to human influenza A viruses during the study period was 14·9%. A(H1N1)pdm09 virus was the dominant (23·1%) subtype detected in pigs by serology followed by the seasonal H1N1 virus (17·3%) and the H3N2 subtype (9·9%). Antibodies against more than one subtype were detected in 132 individual pigs.

Seroprevalence to seasonal H1N1 virus ranged between 2·7% in 2007 and as high as 46·5% in 2008. The prevalence of anti-H3 antibodies in pig sera varied between 0% in 2007 and 33·8% in 2008. Serology to A(H1N1)pdm09 tested positive only in samples collected in 2010. None of the tested sera showed positive antibodies to H5N1 virus. The overall seroprevalence to the viruses tested was notably low in 2006 and 2007 and peaked in 2008 before decreasing in 2009 and 2010 when the peak of A(H1N1)pdm09 was observed (Table 2). The range and mean ± SD of antibody titers to H1N1, H3N2, and A(H1N1)pdm09 viruses are shown in Table 3.

The seroprevalence of H1N1, H3N2, and A(H1N1)pdm09 viruses was compared between pigs ≤4 months old and those >4 months old (Table 4). The seroprevalence of H1N1 and H3N2 viruses was higher in the younger age group (≤4 months old), but the differences were not significant ($P > 0·05$).

The seroprevalence by province of origin of the animals (Banteay Meanchey, Kampong Cham, Kampong Speu, Kampot, Kandal, Prey Veng, Pursat, Svay Rieng, and Takeo provinces) is shown in Figure 2. Evidence of seasonal H1N1 and H3N2 influenza viruses' circulation in pigs was found from eight of the nine provinces from which pigs were sampled (88·9%). All sera originating from Kampot province ($n = 19$) were seronegative to all subtypes. The highest seroprevalence to seasonal H1N1 virus was 52·2% in Banteay Meanchey province. The highest seroprevalence to H3N2 virus was 33·3% in Pursat province and the lowest (0%) in Kampong Speu and Kampot provinces. Samples collected from pigs originating from four provinces (Kandal, Prey Veng, Svay Rieng, and Takeo) were tested for H1N1pdm09 virus by serology after the introduction of the virus in country, and positive results were found in all the four provinces, ranging from 8% in Takeo to 35% in Kandal province.

Discussion

Pigs are susceptible to infection with influenza viruses from mammalian and avian origins.[21] Pigs play an important part in the ecology of influenza A viruses and are a potential source for human pandemic influenza viruses with serious public health implications.[40] According to previous studies, human H1N1 and H3N2 viruses are frequently transmitted to pigs through reverse zoonosis; however, they do not show long-term persistence in pig populations.[41] Nevertheless, the genes of human viruses may persist after reassortment with one or more influenza viruses in pigs.[21] Such circumstances could lead to generation of reassortant viruses with increased cross-species transmissibility, pathogenicity, and lethality, which could cause a human influenza pandemic.

In this study, we performed serological testing for antibodies to influenza A (human H1N1, human H3N2, A(H1N1)pdm09, and avian influenza H5N1) viruses in swine sera collected in Cambodia between 2006 and 2010. No serological tests to detect SIVs were performed. Indeed, SIVs have never been isolated in Cambodia and only rarely in surrounding countries of the region. It should be noted that the HI tests fail to differentiate between A(H1N1)pdm09 and SIVs owing to serologic cross-reactivity in pigs.[33] The average seroprevalence against the human influenza A viruses tested was of 14·9% during the study period. This result is different to those reported in semi-commercial farms in Vietnam (3·1%) and industrial farms in China (61·4%).[29,42]

The highest seroprevalence detected in Cambodian pigs was against the A(H1N1)pdm09 influenza virus followed by the seasonal H1 and the H3 subtypes, respectively. The results also showed evidence that some pigs were exposed to more than one human virus during their short lives. The high levels of A(H1N1)pdm09 virus infections in Cambodian pigs suggest that this strain was widely circulating

Table 2. Annual seroprevalence to each influenza A virus subtypes tested (n = 1147)

Year	No. of sera	HI positivity rate to different influenza virus antigens							
		H1(%)	95%CI	H3(%)	95%CI	A(H1N1) pdm09(%)	95%CI	H5(%)	95%CI
2006	393	5·6	3·5–8·4	2·3	1·1–4·3	NT	–	0*	0·0–12·8
2007	113	2·7	0·6–7·6	0·0	0·0–3·2	NT	–	NT	–
2008	269	46·5	40·4–52·6	33·8	28·2–39·8	NT	–	0*	0·0–7·0
2009	36	19·4	8·2–36·0	13·9	4·7–29·5	0·0	0·0–9·7	0*	0·0–9·7
2010	336	12·5	9·2–16·5	2·4	1·0–4·6	25·6	21·0–30·6	0*	0·0–9·7

*Only a subset of the samples were tested for antibodies against H5N1 virus.
NT, not tested; HI, hemagglutination inhibition assay.

Table 3. HI titers to three different influenza subtypes

Antibody titers	Different influenza subtypes		
	H1N1	H3N2	A(H1N1)pdm09
Range	0–320	0–640	0–640
Mean ± SD	16·2 ± 29·4	10·7 ± 26·7	26·5 ± 59·4
No. of sera tested	1147	1147	372

HI, hemagglutination inhibition assay.

Table 4. Seroprevalence to H1N1, H3N2 viruses in two age groups (n = 538)

Age group	Seropositivity: number positive/number tested (%)			
	H1N1	95%CI	H3N2	95%CI
≤4 month	22/340 (6·5)	4·1–9·6	7/340 (2·1)	0·8–4·2
>4 month	6/198 (3·0)	1·1–6·5	3/198 (1·5)	0·3–4·4
P[a]	0·11		0·75	

[a]Statistical analysis for differences of seropositivity between different age groups; $P < 0·05$ is considered statistically significant.

in the pig population as described in other countries, including Asia.[8,43,44] The potential for concurrent multiple infections with human influenza viruses in pigs needs to be emphasized as it facilitates the opportunity for the generation of new pathogenic variants in pigs through reassortment events, which might then facilitate transmission to humans.[7,45] Moreover, dual infection of A(H1N1)pdm09 and H3N2 viruses in humans was documented in Cambodia.[46]

H5N1 virus has been isolated from pigs in few occasions in Indonesia and China[47–49] with evidence of pig-to-pig transmission in Indonesia,[47] but this virus is still generally considered as poorly transmissible to swine.[40] Antibodies against avian H5 influenza virus were not detected in this study. Our findings, therefore, suggest a low risk of reassortment between avian H5N1 and A(H1N1)pdm09 viruses. Nevertheless, the number of samples tested was limited and the circulation of H5N1 virus in poultry is seasonal and geographically restricted to some provinces. Therefore, H5N1 seroconversions may have been missed.

The overall seroprevalence of each influenza virus subtype detected in pigs follows the human seasonal serotype

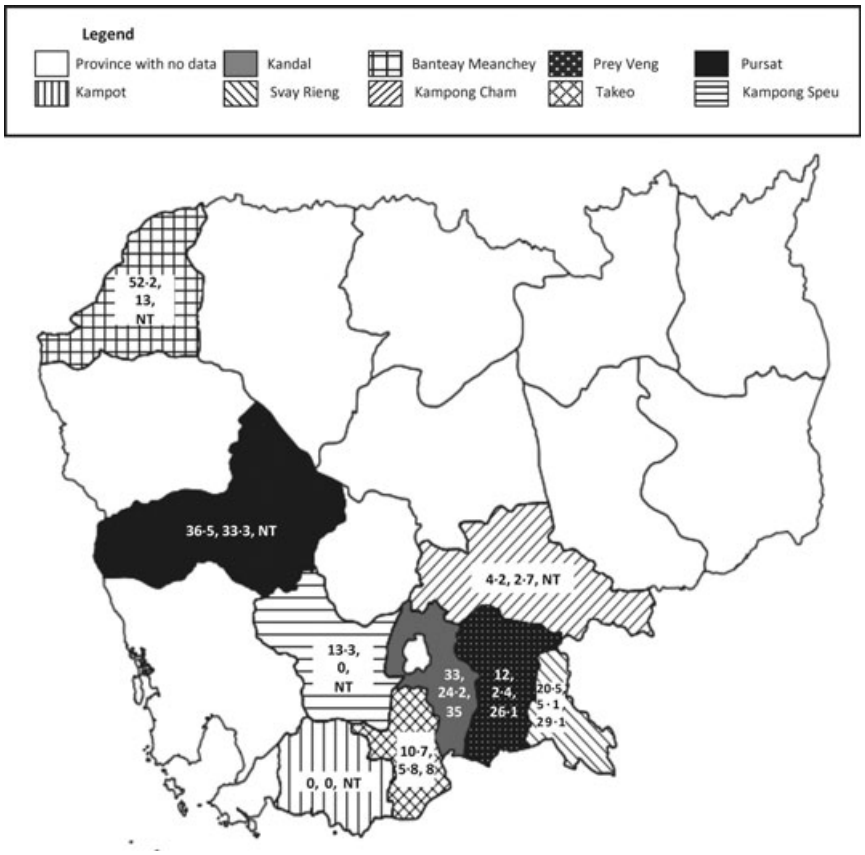

Figure 2. Seroprevalences against H1N1, H3N2, and A(H1N1)pdm09 viruses, respectively, in various provinces in Cambodia.

pattern that was seen in Cambodia during these recent years (IPC unpublished data).[50] The similarity of the seroprevalence in pigs and humans suggests a possible human-to-swine influenza virus transmission in Cambodia. Moreover, the results from our study demonstrated a high seroprevalence to the A(H1N1)pdm09 virus during the post-pandemic period. The situation in the swine population mimics that described for humans where the A(H1N1)pdm09 virus progressively replaced the seasonal H1N1 influenza virus and became the predominant circulating subtype in humans. No samples seropositive against the A(H1N1)pdm09 virus were found during 2009, but the community-level transmission of A(H1N1)pdm09 viruses in human population in Cambodia started only in August 2009 suggesting that only a short delay was required for the transmission from humans-to-swine population. These data also suggest that these positive A(H1N1)pdm09 tests were not a result of cross-reactivity with potentially circulating H1N1 SIVs, as no samples prior to 2010 were seropositive using this test.

Pigs were categorized into two age groups (≤4 months old and >4 months old) as maternal antibodies to influenza viruses can persist for 16 weeks.[51] Only few data on age were available for the pigs that tested positive by serology to A(H1N1)pdm09 virus, and this explains why comparison by age groups was not possible. We found no statistically significant differences in the seroprevalence between the two age groups. This may have resulted from interference by maternal antibodies in the younger age group (≤4 months old), while exposure to the influenza viruses explained the antibody status in the older age group (>4 months old). However, considering the limited number of results compared in each age group, results should be interpreted with care. We did not compare the seroprevalence rates in pigs sampled in farms versus those sampled in slaughterhouse because pigs sent to abattoirs are mostly 10–12 months old, which are older than those living in farms.[52]

In eight of nine provinces, evidence of H1 and H3 influenza virus infections were found. Serologies were surprisingly negative in pigs originating from Kampot, but the low number of samples collected does not allow to draw any conclusion. A(H1N1)pdm09 virus infections were detected in pigs originating from all four provinces sampled after the beginning of the pandemic. This suggests extensive circulation of human influenza virus infections in pigs across Cambodia, although without characterization of the viruses themselves, it cannot be determined whether

there is ongoing circulation of these viruses in swine populations nationally or whether these infections were the result of discrete introductions from human populations with limited onward spread in pigs. The negative results of antibodies to both subtypes in pigs from Kampot may be due to the low number of samples that could influence the seroprevalence. The provinces with high seroprevalences should be investigated further in terms of human influenza cases and human-to-pig interface. Serum samples were mostly collected from a slaughterhouse in Phnom Penh. Pigs are usually slaughtered shortly after arriving in the capital (generally within 24 hours), which does not give sufficient time for a pig to seroconvert following a contamination that occured at the slaughterhouse. Thus, influenza virus contaminations were presumed to have occurred in farms.

Some experimental studies showed that the HI tests are sufficient to differentiate antibodies to H1N1, H3N2, and H1N2 SIV subtypes in European swine.[53,54] However, Kyriakis *et al.*[33] hypothesized that if pigs had been previously infected with, or vaccinated against, European SIVs, they would frequently have serologic cross-reactivity to the A(H1N1)pdm09 virus and related North American SIVs. Hence, sera from pigs either infected or vaccinated with SIVs could have cross-reactive HI antibodies to A(H1N1)pdm09 virus. However, to our knowledge, no autogenous or commercial swine influenza vaccines have been used in the Cambodian swine industry. In addition, no SIVs have been previously isolated in Cambodia. Although a definitive answer would have required to also test each sera for the detection of anti-nucleoprotein antibodies (specific for influenza A), this, along with the lack of positive samples from before 2010, makes the chance of cross-reactivity to A(H1N1)pdm09 in this study unlikely.

As no routine surveillance or systematic surveillance for influenza A in Cambodian pigs has been carried out, this study was started by the IPC through collaboration with the NaVRI. Given the limited resources in establishing nationwide surveillance for influenza in pigs, we considered slaughtered pigs as sentinel pigs to be used to determine the activity of influenza A viruses. The samples from the NaVRI were added up to increase the power of statistic analysis. The results shown here, therefore, do not perfectly represent the entire pig populations in Cambodia because of sampling bias. Majority of samples (682 of 1147) were taken from pigs at the abattoir, which makes difficulty to extrapolate to the whole pig populations in Cambodia. To illustrate, pigs in Cambodia are generally slaughtered at the age of 10–12 months, an overrepresentation of pigs with marketable weight cannot be excluded. However, these results are useful to identify the dominant influenza strains in pigs in the country and to emphasize the urgent need of implementing well-designed surveillance system of influenza A in Cambodian swine population in the nationwide scale.

A more systematic surveillance study needs to be developed and applied for the investigation of influenza A viruses in pig populations in Cambodia. Further, studies to collect and characterize viruses as well as using molecular techniques to detect, monitor, and evaluate the persistence of circulating strains of influenza viruses in pig's farms rather than in abattoirs where probably only apparently healthy animals are slaughtered are recommended to identify their future evolution and ensure early detection of potentially pandemic strains. Participation at the community level needs to be incorporated into the existing surveillance for influenza viruses in Cambodian pigs to enhance the sensitivity of detecting influenza cases in swine.

Conclusion

This study provides the first data on sustained human influenza virus infections in pigs in Cambodia. Serological surveillance results indicated that seasonal H1, H3, and A(H1N1)pdm09 subtypes were common in Cambodian pigs and probably resulted from extensive transmission of influenza A virus from humans back to pigs. On the other hand, infection with the H5 subtype was not detected. Serological investigation of influenza viruses may give useful information for surveillance of novel influenza viruses in pigs. Further, molecular surveillance is required for the study of genetic components of influenza viruses to closely monitor their characterization, their extent of reassortment, and their potential impact on public health.

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Disclaimer

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References

1 Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology 1993; 193:503–506.
2 Ma W, Kahn RE, Richt JA. The pig as a mixing vessel for influenza viruses: human and veterinary implications. J Mol Genet Med 2009; 3:158–166.
3 Scholtissek C, Stech J, Krauss S, Webster RG. Cooperation between the hemagglutinin of avian viruses and the matrix protein of human influenza A viruses. J Virol 2002; 76:1781–1786.
4 Garten RJ, Davis CT, Russell CA *et al.* Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 2009; 325:197–201.
5 Peiris JS, Poon LL, Guan Y. Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. J Clin Virol 2009; 45:169–173.
6 Smith GJ, Vijaykrishna D, Bahl J *et al.* Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 2009; 459:1122–1125.
7 Zhu H, Zhou B, Fan X *et al.* Novel reassortment of eurasian avian-like and pandemic/2009 influenza viruses in Swine: infectious potential for humans. J Virol 2011; 85:10432–10439.
8 Song MS, Lee JH, Pascua PN *et al.* Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea. J Clin Microbiol 2010; 48:3204–3211.
9 Howden KJ, Brockhoff EJ, Caya FD *et al.* An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can Vet J 2009; 50:1153–1161.
10 Brookes SM, Brown IH. A/H1N1/pdm09 virus: dynamics of infection in pigs and people. Vet Rec 2011; 169:151–152.
11 Pasma T, Joseph T. Pandemic (H1N1) 2009 Infection in Swine Herds, Manitoba, Canada. Emerg Infect Dis 2010; 16:706–708.
12 Pereda A, Cappuccio J, Quiroga MA *et al.* Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis 2010; 16:304–307.
13 Lange E, Kalthoff D, Blohm U *et al.* Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs. J Gen Virol 2009; 90:2119–2123.
14 Brookes SM, Irvine RM, Nunez A *et al.* Influenza A (H1N1) infection in pigs. Vet Rec 2009; 164:760–761.
15 Brookes SM, Brown IH. A/H1N1/pdm09 virus: dynamics of infection in pigs and people. Vet Rec 2011; 169:151–152.
16 Ducatez MF, Hause B, Stigger-Rosser E *et al.* Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerg Infect Dis 2011; 17:1624–1629.
17 Sun D, Schwartz K, Janke G, Haney D, Block S, Yoon K. Occurrence of novel reassortants between North American swine influenza virus and pandemic 2009 H1N1 virus in US swine. American Society for Virology 30th Annual Meeting; University of Minnesota, Minneapolis 2011; 221.
18 Starick E, Lange E, Fereidouni S *et al.* Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. J Gen Virol 2011; 92:1184–1188.
19 Howard WA, Essen SC, Strugnell BW *et al.* Reassortant Pandemic (H1N1) 2009 virus in pigs, United Kingdom. Emerg Infect Dis 2011; 17:1049–1052.
20 Kitikoon P, Sreta D, Na Ayudhya SN *et al.* Brief report: molecular characterization of a novel reassorted pandemic H1N1 2009 in Thai pigs. Virus Genes 2011; 43:1–5.
21 Moreno A, Di Trani L, Faccini S *et al.* Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. Vet Microbiol 2011; 149:472–477.
22 Vijaykrishna D, Poon LL, Zhu HC *et al.* Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science 2010; 328:1529.
23 CDC. Update: influenza A (H3N2)v transmission and guidelines – Five States, 2011. MMWR 2012; 60:1741–1744.
24 Huynh TTT, Aarnink AJA, Drucker A, Verstegen MWA. Pig production in Cambodia, Laos, Philippines, and Vietnam: a review. Asian J Agric Dev 2007; 4:69–90.
25 Samkol P, Borin K, Sovann S. Pig Systems in Southeast Asia – The Case of Cambodia. International Livestock Research Institute. Available at: http://ilri.org/InfoServ/Webpub/fulldocs/Pig%20Systems_proceeding/CH_04_Borin_Sovann.pdf (accessed 15 January 2012).
26 Chetra S, Bourn D. Review of animal production in Cambodia. The sub-regional environmental animal health management initiative for enhanced smallholder production in South East Asia. 2009; 1–30.
27 Dietze K, Pinto J, Wainwright S, Hamilton C, Khomenko S. Porcine reproductive and respiratory syndrome (PRRS) virulence jumps and circulation in Southeast Asia. Focus on Porcine reproductive and respiratory syndrome (persistent PRRS). 2011; 1–8.
28 Palese P. Influenza: old and new threats. Nat Med 2004; 10:S82–S87.
29 Trevennec K, Cowling BJ, Peyre M *et al.* Swine influenza surveillance in East and Southeast Asia: a systematic review. Anim Health Res Rev 2011; 12:213–223.
30 Van Reeth K, Nicoll A. A human case of swine influenza virus infection in Europe-implications for human health and research. Euro Surveill 2009; 14:19124.
31 OFFLU. Technical meeting – coordinating worldwide surveillance for influenza in pigs. 2011. OIE, FAO, Paris, France, 6–7 April 2011. Available at http://www.offlu.net/fileadmin/home/en/meeting-reports/pdf/SIV-Summary.pdf (Accessed 15 December 2011).
32 Ramsay G, Andrews L, Hoffmann D. Poverty Alleviation and Food Security in Asia: The Role of Livestock Bangkok, Thailand. FAO. RAP Publication 1999/4. UNDP/FAORAS/95/01T. P. 1–2, 19.
33 Kyriakis CS, Olsen CW, Carman S *et al.* Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe. Emerg Infect Dis 2010; 16:96–99.
34 WHO. Manual for the laboratory diagnosis and virological surveillance of influenza. 2011; Available at http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf (Accessed 5 December 2011).
35 Chambers TM, Hinshaw VS, Kawaoka Y, Easterday BC, Webster RG. Influenza viral infection of swine in the United States 1988–1989. Arch Virol 1991; 116:261–265.
36 Olsen CW, Carey S, Hinshaw L, Karasin AI. Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States. Arch Virol 2000; 145:1399–1419.
37 Jung K, Song D-S, Kang B-K, Oh J-S, Park B-K. Serologic surveillance of swine H1 and H3 and avian H5 and H9 influenza A virus infections in swine population in Korea. Prev Vet Med 2007; 79:294–303.
38 Reed L, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938; 27:493–497.
39 Ross TD. Accurate confidence intervals for binomial proportion and Poisson rate estimation. Comput Biol Med 2003; 33:509–531.
40 Lipatov AS, Kwon YK, Sarmento LV *et al.* Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. PLoS Pathog 2008; 4:e1000102.
41 Van Reeth K. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet Res 2007; 38:243–260.
42 Liu W, Wei MT, Trong Y *et al.* Seroprevalence and genetic characteristics of five subtypes of influenza A viruses in the Chinese pig population: a pooled data analysis. Vet J 2011; 187:200–206.
43 Forgie SE, Keenliside J, Wilkinson C *et al.* Swine outbreak of pandemic influenza A virus on a Canadian research farm

supports human-to-swine transmission. Clin Infect Dis 2011; 52:10–18.

44 Sreta D, Tantawet S, Na Ayudhya SN *et al.* Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. Emerg Infect Dis 2010; 16:1587–1590.

45 Yoon K-J, Janke BH, Morilla A, Zimmerman JJ. Swine Influenza: Etiology, Epidemiology, and Diagnosis. Trends in Emerging Viral Infections of Swine. Ames: Iowa State Press, 2008; 23–28.

46 Myers CA, Kasper MR, Yasuda CY *et al.* Dual infection of novel influenza viruses A/H1N1 and A/H3N2 in a cluster of Cambodian patients. Am J Trop Med Hyg 2011; 85:961–963.

47 Nidom CA, Takano R, Yamada S *et al.* Influenza A(H5N1) viruses from pigs, Indonesia. Emerg Infect Dis 2010; 16:1515–1523.

48 Takano R, Nidom CA, Kiso M *et al.* A comparison of the pathogenicity of avian and swine H5N1 influenza viruses in Indonesia. Arch Virol 2009; 154:677–681.

49 Zhu Q, Yang H, Chen W *et al.* A naturally occuring deletion in its NS gene contributes to the attenuation of an H5N1 swine influenza virus in chickens. J Virol 2008; 82:220–228.

50 Mardy S, Ly S, Heng S *et al.* Influenza activity in Cambodia during 2006–2008. BMC Infect Dis 2009; 9:168.

51 Easterday BC, Van Reeth K. Swine influenza; in Straw BE, D'Allaire S, Mengeling WL, Taylor DJ (eds): Diseases of Swine, 8th edn. Ames, IA, Iowa State University Press, 1999; 277–290.

52 Duong V, Sorn S, Holl D, Rani M, Deubel V, Buchy P. Evidence of Japanese encephalitis virus infections in swine populations in 8 provinces of Cambodia: implications for national Japanese encephalitis vaccination policy. Acta Trop 2011; 120:146–150.

53 Reeth KV, Brown I, Essen S, Pensaert M. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus Res 2004; 103:115–124.

54 Van Reeth K, Labarque G, Pensaert M. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. Viral Immunol 2006; 19:373–382.